# Lesions and Distribution of Viral Antigen Following an Experimental Infection of Young Seronegative Calves with Virulent Bovine Virus Diarrhea Virus-Type II

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## ABSTRACT

**During the past several years, acute infections with bovine viral diarrhea virus (BVDV) have been causally linked to hemorrhagic and acute mucosal disease-like syndromes with high mortality. The majority of BVDVs isolated in such cases have been classified as type II on the basis of genetic and antigenic characteristics. It was our objective to examine clinical disease, lesions and potential sites of viral replication, following experimental BVDV type II infection in young calves. On approximately day 35 after birth, calves that had received BVDV-antibody-negative colostrum were infected by intranasal inoculation of $5 \times 10^5$ $TCID_{50}$ of BVDV type II isolate 24515 in 5 mL of tissue culture fluid (2.5 mL/nostril). Calves were monitored twice daily for signs of clinical disease. Approximately 48–72 h after infection, all calves developed transient pyrexia (39.4–40.5°C) and leukopenia. Beginning on approximately day 7 after infection, all calves developed watery diarrhea, pyrexia (40.5–41.6°C), marked leukopenia (≥ 75% drop from preinoculation values), variable thrombocytopenia, and moderate to severe depression. Calves were euthanized on days 10, 11, or 12 after infection due to severe disease. Gross and histological lesions consisted of multifocal bronchointerstitial pneumonia (involving 10%–25% of affected lungs), bone marrow hypoplasia and necrosis, and minimal erosive lesions in the alimentary tract. Immunohistochemical staining for BVDV revealed widespread viral antigen usually within epithelial cells, smooth muscle cells and mononuclear phagocytes in multiple organs, including lung, Peyer's patches, gastric mucosa, thymus, adrenal gland, spleen, lymph nodes, bone marrow, and skin. This BVDV type II isolate caused rapidly progressive, severe multisystemic disease in seronegative calves that was associated with widespread distribution of viral antigen and few gross or histological inflammatory lesions.**

## RÉSUMÉ

**Au cours des dernières années, un lien causal a été établi entre les infections aiguës avec le virus de la diarrhée virale bovine (VDVB) et des syndromes hémorragiques et aigus de la maladie des muqueuses associée à une forte mortalité. La majorité des isolats de VDVB retrouvés dans ces cas furent classifiés de type II sur la base de leurs caractéristiques génétiques et antigéniques. L'objectif ce cette étude était d'examiner la maladie clinique, les lésions et les sites potentiels de réplication virale suite à l'infection expérimentale de jeunes veaux par du VDVB de type II. Environ 35 jours après la naissance, des veaux ayant reçu du colostrum ne contenant pas d'anticorps contre le VDVB furent infectés par voie intranasale avec $5 \times 10^5$ $TCID_{50}$ de l'isolat 24515 du VDVB de type II dans 5 mL de milieu de culture cellulaire (2,5 mL par narine). Les veaux furent observés deux fois par jour pour vérifier la présence de signes cliniques. Environ 48–72 h après l'infection tous les veaux ont développé une hyperthermie transitoire (39,4–40,5 °C) et une leucopénie. Tous les veaux ont développé une diarrhée aqueuse, de l'hyperthermie (40,5–41,6 °C), une leucopénie marquée (diminution de ≥ 75 % par rapport aux valeurs pré-inoculation), une thrombocytopénie variable et une dépression modérée à sévère environ 7 j après l'infection. Les veaux furent euthanasiés aux jours 10, 11 ou 12 post-infection due à leur pauvre état de santé. Les lésions macroscopiques et histologiques observées consistaient en une broncho-pneumonie multifocale impliquant environ 10–25 % des poumons, une hypoplasie et une nécrose de la moelle osseuse, et de minimes lésions érosives dans le tractus digestif. L'examen immunohistochimique pour la présence de VDVB permit de mettre en évidence une dissémination généralisée de l'antigène viral à l'intérieur de cellules épithéliales, de cellules musculaires lisses et de phagocytes mononucléaires dans plusieurs organes dont les poumons, les plaques de Peyer, la muqueuse gastrique, le thymus, les glandes surrénales, la rate, les ganglions lymphatiques, la moelle osseuse et la peau. Cet isolat de type II du VDVB cause rapidement une maladie multisystémique sévère progressive chez des veaux séronégatifs et fut associé avec la distribution rapide d'antigène viral mais causa peu de lésions inflammatoires macroscopiques ou histologiques.**

***(Traduit par docteur Serge Messier)***

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## INTRODUCTION

In 1946, Olafson and colleagues reported an apparently new disease in cattle, "virus diarrhea," characterized by high fever, leukopenia and severe ulceration of the alimentary tract (1,2). This disease was further described as being acute and contagious. Subsequently, bovine virus diarrhea virus (BVDV) has been implicated in numerous clinical syndromes in cattle, including reproductive failure, fetal defects, and "mucosal disease" resulting from persistent BVDV infection following intrauterine exposure during the first trimester of gestation (3). However, it was generally thought that most BVDV infections in cattle were subclinical and followed by complete recovery and the development of protective immunity (3).

In the late 1980's BVDVs were causally linked to acute, hemorrhagic disease with high morbidity and mortality in affected herds (3). More recently, noncytopathic BVDVs were isolated from cattle dying with acute mucosal disease-like syndromes (5,6). There have been several epidemics of acute BVDV-associated disease in the northeastern US and Canada with death losses approaching 100% in some groups of young veal calves (3,5–7). Recently the extent of genetic and antigenic diversity of the highly mutable pestiviruses have been reexamined (7,8). BVDVs have been classified into types I and II on the basis of sequence differences in the 5′ untranslated region of the viral genome and phenotypic analysis with panels of monoclonal antibodies (8). Most of the virulent isolates from recent cases of acute BVDV in North America have been classified as type II (3,5,8).

Although there is a considerable body of literature concerning the pathogenesis and lesions of persistent BVDV infection (9), there is comparatively little information on the pathogenesis of, and extent of systemic viral replication in acute, virulent BVDV infections, including type II BVDVs (5,10–12). It was our objective to examine the lesions and sites of viral replication following experimental BVDV type II infection in young calves similar to those dying in recent epidemics.

## MATERIALS AND METHODS

### CALVES AND EXPERIMENTAL DESIGN

Eight Holstein and Holstein-beef cross neonatal bull calves were the subjects of this study and were treated in accordance with the *Guidelines For the Care and Use of Experimental Animals* established by the Canadian Council on Animal Care. Calves received maternal colostrum or were prevented from suckling the dam and were fed pooled colostrum that tested negative for BVDV-specific antibodies on the basis of an ELISA (13) using a solubilized (14) antigen prepared from cells infected with the NADL strain of BVDV. All calves tested BVDV-negative and BVDV-specific-antibody-negative prior to inclusion in the study.

Calves were removed from dams shortly after birth, housed in isolation facilities, and maintained on commercial milk replacer (4 L/d) and ad libitum alfalfa-based pelleted feed and alfalfa-grass hay. On approximately day 35 after birth, 6 calves were infected by intranasal inoculation of $5 \times 10^5$ $TCID_{50}$ of BVDV in 5 mL of tissue culture fluid (2.5 mL/nostril). Two calves were sham inoculated controls. Calves were monitored twice daily for signs of clinical disease. Rectal temperatures were recorded twice daily and complete blood counts were determined on days 0, 1, 3, 5, 7, 9, and on days 10, 11, or 12, after infection. For humane reasons, calves that had at least 3 of the 4 following signs for more than 2 consecutive d were euthanized by barbiturate overdose: rectal temperatures $\geq$ 40.6°C, watery diarrhea, total white cell counts $\leq$ 2000 mL, or marked depression.

### BVDV

Noncytopathic BVDV isolate No. 24515 was obtained from the tissues of a fetus from a herd in Ontario, that experienced acute "mucosal disease-like" syndrome, gastrointestinal disease, respiratory disease, and death of calves and cows. This isolate was classified as BVDV type II on the basis of antigenic and genetic analyses (5). Virus for experimental infection was propagated in low passage (< 10) cultures of embryonic bovine turbinate cells and virus passages 7 and 8 were used for calf infections.

### VIRUS ISOLATION

Jugular blood was collected in heparinized tubes. Two mL of blood were mixed with 13 mL of lysis buffer containing ammonium chloride and centrifuged. The resulting pellet of leukocytes was washed once in sterile phosphate buffered saline, resuspended in 1 mL of modified Eagle's medium and stored frozen at −70°C. Two hundred μL aliquots of thawed, mixed samples were cultured with embryonic bovine turbinate cells (passage $\leq$ 10) in 24 well tissue culture plates for 7 d. Cultures were freeze-thawed once and 75 μL of supernatant was co-cultured with approximately $10^4$ Madin-Darby bovine kidney cells, in 100 μL in duplicate, in 96-well tissue culture plates. After 4 d, medium was removed, the cell monolayers were fixed in acetone and stained for the gp48 antigen of BVD virus using a monoclonal antibody (ascites, IgG2b isotype), 15C5 (15) and an avidin-biotin immunoperoxidase technique (16). Each 96-well plate contained wells that were inoculated with 25 μL of serum from a BVDV-persistently-infected calf (positive control) and uninoculated cells (negative control).

### HEMATOLOGY

Complete blood cell counts were performed using an automated cell counter (Baker System 9000 Hematology Series Cell Counter, Seronon Baker Diagnostics, Allentown, Pennsylvania, USA). Manual platelet counts were performed on blood samples (Unopette Microcollection System, Becton-Dickinson, Rutherford, New Jersey, USA) from calves numbers 2, 3, and 6 using a hemacytometer (Bright-Line Hemacytometer, American Optical, Scientific Instrument Division, Buffalo, New York, USA). Plasma protein concentration was determined by refractometry. Plasma fibrinogen concentration was determined by the heat percipitation technique, with fibrinogen concentration being the difference between plasma protein concentration and plasma protein concentration after heating plasma at 58°C for 3 min.

### HISTOLOGY AND IMMUNOHISTOCHEMISTRY

Tissue sections were prepared from paraffin blocks of formalin-fixed

**TABLE I. Analysis of terminal (premortem) blood and bone marrow (postmortem) from calves**

| Parameter | Calf No. 2 | Calf No. 3 | Calf No. 6 | Calf No. 7 | Calf No. 2290 | Calf No. 6395 | Reference Values* |
|---|---|---|---|---|---|---|---|
| Total leukocyte count ($\times 10^9$/L) | 1.9 | 4.5 | 4.4 | 1.40 | 2.20 | 2.2 | 7.67–13.8 |
| Segmented neutrophils ($\times 10^9$/L) | 0.114 | 1.035 | 0.440 | 0.098 | 0.286 | 0.308 | 1.451–4.203 |
| Band neutrophils ($\times 10^9$/L) | 0.019 | 0.495 | 0.440 | 0.070 | 0.176 | 0.198 | 0–0.080 |
| Metamyelocyte neutrophils ($\times 10^9$/L) | — | — | — | — | — | — | — |
| Eosinophils ($\times 10^9$/L) | — | — | — | — | — | — | 0–0.448 |
| Lymphocytes ($\times 10^9$/L) | 1.710 | 2.880 | 2.904 | 1.190 | 1.694 | 1.672 | 4.682–9.040 |
| Monocytes ($\times 10^9$/L) | 0.057 | 0.090 | 0.616 | 0.042 | 0.022 | 0.022 | 0.524–1.064 |
| Toxic change | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | — |
| Platelets ($\times 10^9$/L) | < 100‡ | 20 | 167 | 103 | 121 | < 10 | 192–892 |
| Erythrocyte count ($\times 10^{12}$/L) | 9.10 | 10.25 | 6.85 | 6.30 | 8.73 | 8.25 | 8.50–10.5 |
| Hemoglobin (g/L) | 92 | 101 | 75 | 69 | 90 | 87 | 97–127 |
| Hematocrit (L/L) | 0.299 | 0.316 | 0.225 | 0.206 | 0.280 | 0.266 | 0.32–0.40 |
| Mean corpuscular volume (fL) | 32.9 | 30.8 | 32.8 | 32.7 | 32.1 | 32.3 | 34.6–41.0 |
| Mean corpuscular hemoglobin (Pg) | 10.1 | 9.9 | 10.9 | 11.0 | 10.3 | 10.5 | 10.2–13.4 |
| Mean corpuscular hemoglobin concentration (g/L) | 308 | 320 | 333 | 335 | 321 | 327 | 284–340 |
| Plasma proteins (g/L) | 56 | 48 | 46 | 45 | 48 | 45 | 56–68 |
| Fibrinogen (g/L) | 6 | 3 | 5 | 6 | 4 | 4 | 0–2.8 |
| Protein : Fibrinogen ratio | 8:1 | 15:1 | 8:1 | 7:1 | 11:1 | 10:1 | > 15:1 |
| Bone marrow | hypoplasia | hypoplasia necrosis | hypoplasia | ND† | hypoplasia necrosis | hypoplasia | — |
| Day of euthanasia | 12 | 10 | 11 | 10 | 10 | 10 | |

*Reference values for 3–16 wk old calves from: Jain NC. Comparative hematology of common domestic animals. In: Essentials of Veterinary Hematology. Lea & Febiger: Philadelphia, Pennsylvania, 1993: 26.
† ND — not done
‡ Aged blood sample (2 d old) precluded automated platelet count; Platelet count estimated from blood smear
§ Not applicable due to platelet clumping

tissue and stained for the presence of the BVDV using the previously described immunohistochemical procedure, adapted for a robotic slide stainer (16,17). Serial sections from each tissue block were cut, affixed to slides coated with a tissue adhesive, digested with proteolytic enzymes to counteract the effects of the fixative on tissue and viral antigens, and treated to inactivate endogenous peroxidases. Slides were stained immunohistochemically for BVDV using the monoclonal antibody, 15C5 and an avidin-biotin-complex immunoperoxidase method (15,17). Controls for the specificity of the staining procedure included serial sections from each tissue block concurrently stained using an identical procedure, but substituting an irrelevant isotype-matched monoclonal antibody for 15C5. In addition, tissues from 2 clinically-normal, BVDV-negative, BVDV-seronegative, age-matched, sham-inoculated control calves were examined. Positive controls for the staining procedure included concurrent staining of the suspect tissue specimens with tissues from animals proven to have succumbed to BVDV-induced mucosal disease. All tissues were examined, blindly, by an observer.

## RESULTS

### CLINICAL RESPONSES/VIRUS ISOLATION

Approximately 48–72 h after infection, all 6 calves developed transient pyrexia (39.4–40.5°C). Beginning 6–8 d after infection all calves developed unremitting pyrexia (40.5–41.6°C), that was accompanied by watery diarrhea, and moderate to severe depression. Calves were euthanized on day 10 ($n = 4$), 11 ($n = 1$), or 12 ($n = 1$) after infection due to severe clinical disease. One calf (No. 2) developed convulsions prior to euthanasia on day 12. Bovine virus diarrhea virus was isolated from buffy coat leukocytes from all calves on 2 or more days after infection.

### CLINICAL PATHOLOGIC FINDINGS

Coincident with the 1st episode of pyrexia there was transient leukopenia (approximately 50% of preinoculation values). Beginning approximately 7–8 d after infection there was progressive marked leukopenia (approximately 25% of preinoculation values) that was further characterized by neutropenia with a left shift to metamyelocytes, toxic changes, lymphopenia, anemia, thrombocytopenia, hypoproteinemia and hyperfibrinogenemia (Table I). Examination of bone marrow collected at necropsy revealed that granulopoiesis was minimal in all calves, with immature granulocytic cells predominating.

Thrombopoiesis was depressed in all cases, except in calf No. 6, in which mature, but especially immature, megakaryocytes were present in relatively high numbers in the bone marrow. Anemia was mild to moderate, microcytic, normochromic and nonregenerative in all cases. Bone marrow necrosis was observed in 2 out of 5 calves examined (No. 3, No. 2290). Necrotic bone marrow was characterized by areas of indistinct morphology, karyorrhexis, and phagocytosis of debris by large mononuclear cells. In 3 of 5 calves there was viral antigen (No. 3, No. 6, No. 6395) in stromal cells in the bone marrow. In 2 of these calves (No. 6, No. 6395), there was multifocal BVD viral antigen suggestive of viral replication in megakaryocytes.

### PATHOLOGY AND IMMUNOHISTOCHEMISTRY

*Alimentary tract* — Gross lesions in the alimentary tract were generally minimal in all 6 calves. Multifocally, in all 6 calves, viral antigen was apparent in squamous epithelium throughout the oral cavity (Fig. 1). One calf (No. 6395) had an 8 cm area

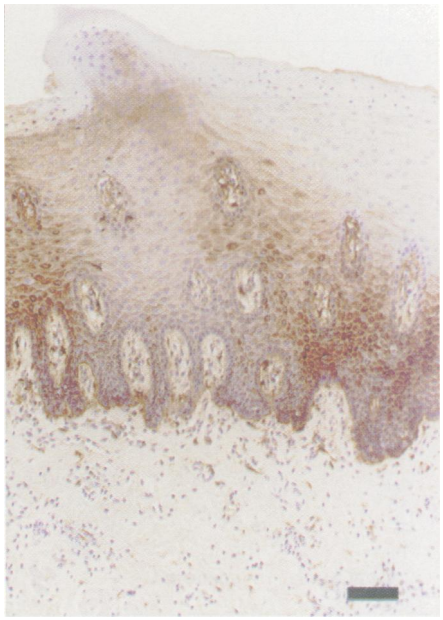

**Figure 1. Photomicrograph of oral mucosa calf No. 6395. Bovine viral diarrhea virus antigen in the cytoplasm of squamous epithelial cells. ABC immunoperoxidase stain for BVDV; Bar = 100 μm.**

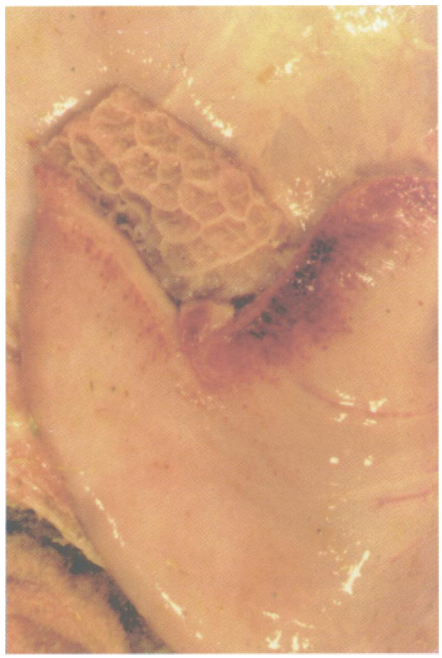

**Figure 2. Reticulum calf No. 6395. There is subserosal hemorrhage.**

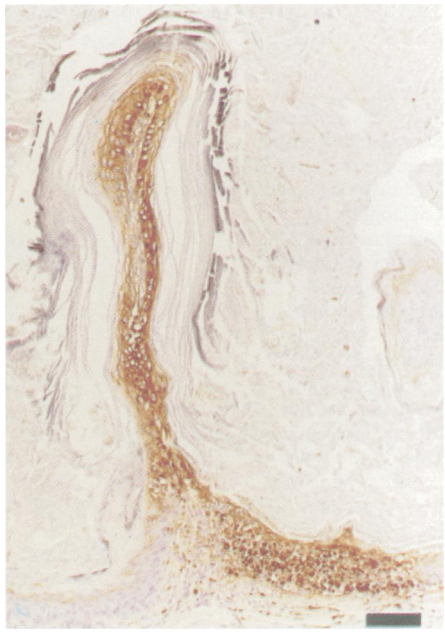

**Figure 3. Photomicrograph of reticulum calf No. 6395. Bovine viral diarrhea virus antigen in the cytoplasm of squamous epithelial cells. Note degeneration of infected cells and absence of inflammatory response. ABC immunoperoxidase stain for BVDV; Bar = 100 μm.**

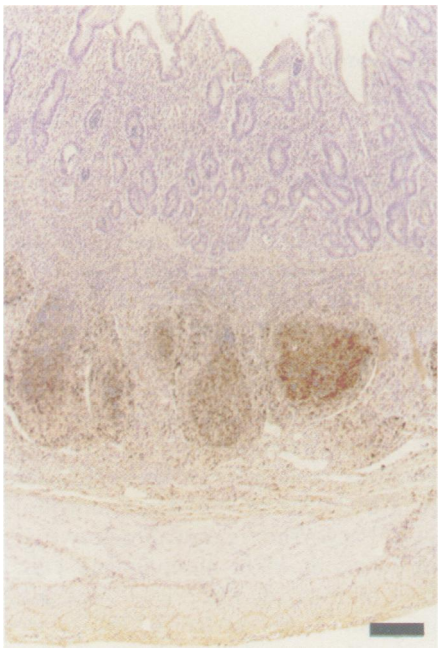

**Figure 4. Photomicrograph of ileum calf No. 7. Bovine viral diarrhea virus antigen in an ileal Peyer's patch that is depleted of lymphocytes. There is minimal inflammation in crypts in enteric mucosa. ABC immunoperoxidase stain for BVDV; Bar = 200 μm.**

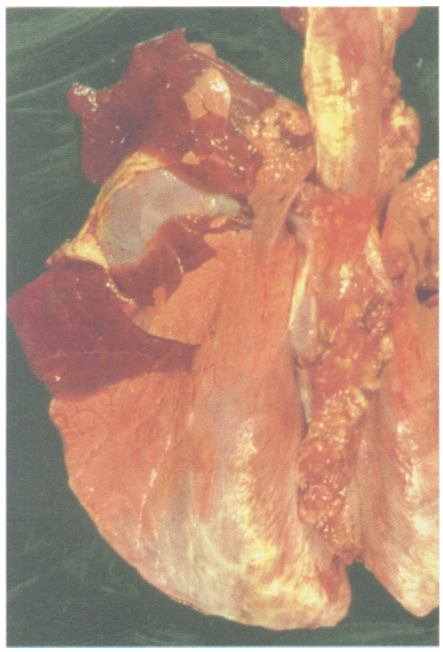

**Figure 5. Lung calf No. 2290. There is consolidation in anterior-ventral areas of lung lobes.**

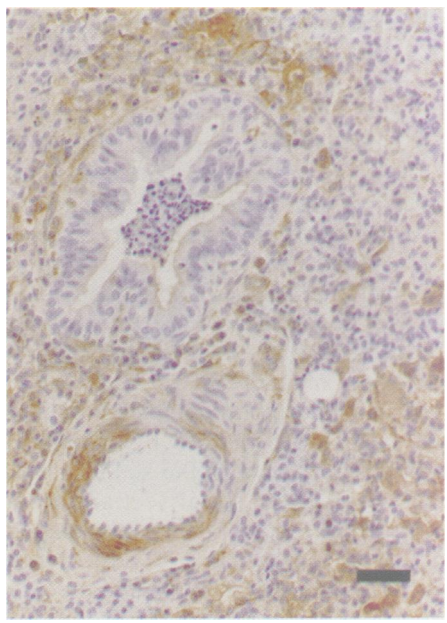

**Figure 6. Photomicrograph of lung calf No. 2290. Bovine viral diarrhea virus antigen in the cytoplasm of alveolar macrophages and smooth muscle cells in the tunica muscularis of an arteriole. ABC immunoperoxidase stain for BVDV; Bar = 50 μm.**

of suffusive subserosal hemorrhage and focal erosion in the mucosa of the reticulum (Fig. 2). No hemorrhages were apparent in the other 5 calves. Viral antigen was apparent multifocally in the mucosa of the forestomachs of all 6 calves (Fig. 3). Two calves (Nos. 3 and 6) had mild hyperemia of the small intestine. One calf (No. 3) had linear hyperemia of the mucosa of the spiral colon. Infrequently, there was necrosis of epithelial cells, erosion, and mild to moderate acute inflammation in the oral cavity and forestomachs, which was associated with the presence of BVDV antigen. Multifocally, in the abomasum there was viral antigen in the glandular epithelium, usually unaccompanied by any histological lesions. Multifocally and segmentally, there was viral antigen in smooth muscle in muscularis mucosa, tunica muscularis, and tunica media of blood

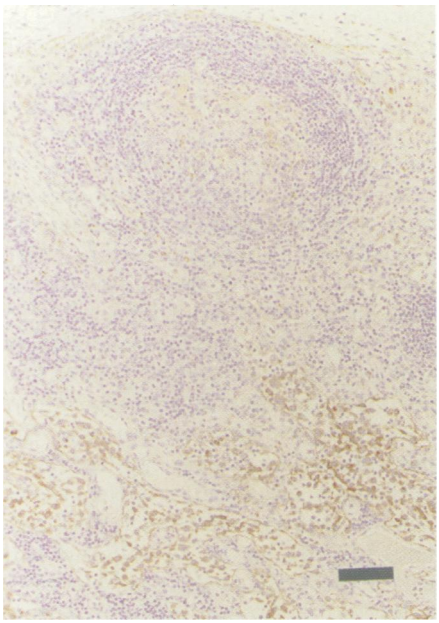

**Figure 7. Photomicrograph of mesenteric lymph node calf No. 6. Bovine viral diarrhea virus antigen in the cytoplasm of macrophages lining sinuses, intrafollicular dendritic cells and tingible body macrophages in cortical follicles and in macrophages in the paracortex. ABC immunoperoxidase stain for BVDV; Bar = 100 μm.**

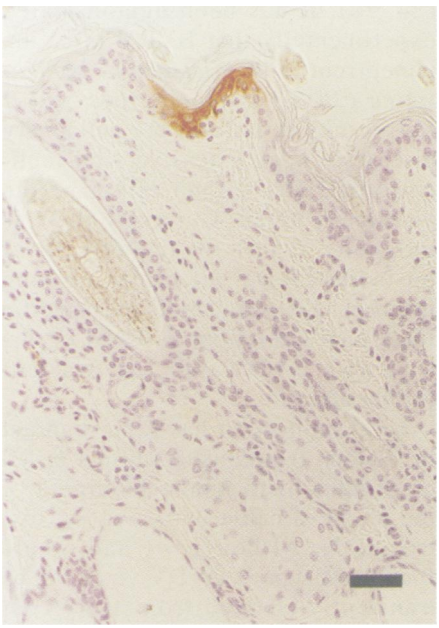

**Figure 10. Photomicrograph of interdigital skin calf No. 6395. Bovine viral diarrhea virus antigen in the cytoplasm of squamous epithelial cells. ABC immunoperoxidase stain for BVDV; Bar = 100 μm.**

vessels in the forestomachs, abomasum and intestines (Fig. 4). There was minimal or no evidence of viral antigen in the mucosal epithelium or glands of the intestines (Fig. 4). Consistently, in all 6 calves, BVDV antigen was prominent in Peyer's patches in the ileum. Virus was present in stromal (nonlymphoid) cells, but not evident in lymphocytes (Fig. 4). The presence of BVDV antigen was associated with variable depletion of the lymphocytes compared to clinically normal calves. In one calf (No. 2290), there was widespread viral replication in Kupffer cells in the liver, and in another calf (No. 6), there was multifocal viral replication in acinar cells the exocrine pancreas.

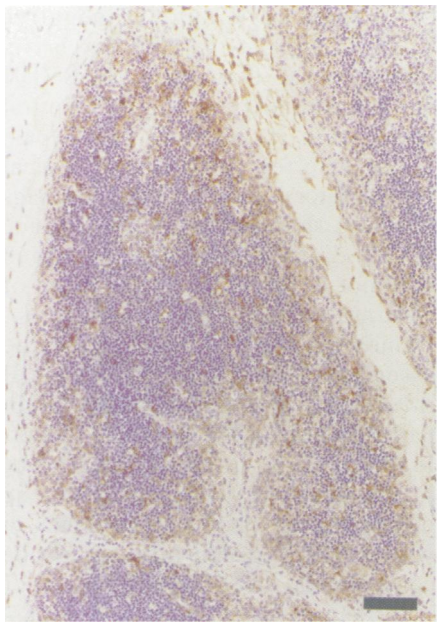

**Figure 8. Photomicrograph of thymus calf No. 2. Bovine viral diarrhea virus antigen in the cytoplasm of large pleomorphic cells in the cortex and medulla. ABC immunoperoxidase stain for BVDV; Bar = 100 μm.**

*Respiratory tract* — Gross lesions consisted of multifocal areas of consolidation, ranging from approximately 10% to 25% of affected lungs in all 6 calves (Fig. 5). A mixed population of bacteria, including *Pasteurella haemolytica* and *Actinomyces pyogenes*, were isolated in low numbers from one of 2 lungs cultured (No. 6395).

In 4 of the 6 calves (Nos. 2, 6, 7 and 2290) there was viral antigen in alveolar macrophages (Fig. 6). All calves had some degree of bronchointerstitial pneumonia or bronchopneumonia, however in only one calf (No. 2) was there BVDV antigen in bronchiolar epithelium. In another calf (No. 2290) there was viral antigen focally in the tunica muscularis of arterioles (Fig. 6). Virus was not apparent in tissue from 2 of 2 nasal turbinates examined.

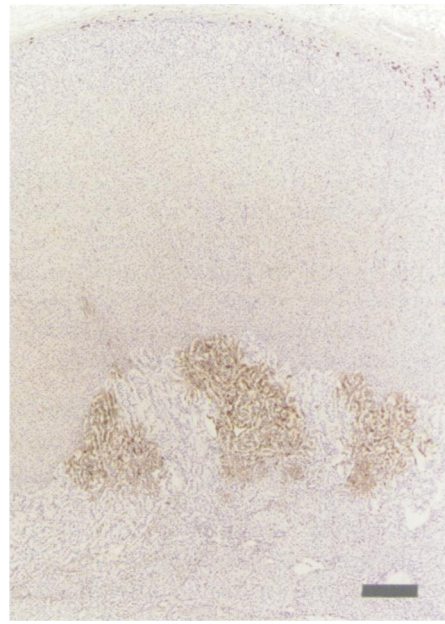

**Figure 9. Photomicrograph of adrenal gland calf No. 2290. Bovine viral diarrhea virus antigen in the cytoplasm of cells in the zona reticularis. ABC immunoperoxidase stain for BVDV; Bar = 200 μm.**

*Lymphoid tissues* — In the mesenteric and bronchial lymph nodes, there was a variable degree of viral antigen that was largely confined to the cytoplasm of macrophages in the medullary sinuses (Fig. 7). There was scant evidence of viral antigen in tingible body macrophages and intrafollicular dendritic cells in follicles (Fig. 7). In the paracortex, scant viral antigen was present in macrophages (Fig. 7), and there was no convincing evidence of viral antigen in lymphocytes (Fig. 7). Neither was there viral antigen apparent in medullary B cell cords. In both the cortex and paracortex, there was moderate depletion of lymphocytes compared to similar sections from clinically normal calves. Viral antigen was commonly seen focally in the tunica muscularis of arterioles in the hilus of lymph nodes. In the spleens of 3 of the 7 calves (Nos. 2, 3 and 6) BVDV antigen was present in scattered fixed mononuclear phagocytes in the red pulp. In 2 of the 7 calves (Nos. 2 and 6) there was also prominent staining of mononuclear phagocytes in the marginal zone of the spleen. There was virtually no staining for viral antigen in the follicles or periarteriolar sheaths, the B- and T-lymphocyte-dependent zones, respectively. In the thymuses of all 6 calves there was

viral antigen in epithelial cells in the cortex, and a mixed population of large epithelial cells in the medulla, including Hassal's corpuscles (Fig. 8).

*Central nervous system* — There was no viral antigen nor were there lesions apparent in representative sections of cerebral cortex, brain stem, or cerebellum in any of the 6 calves examined, including the one calf that had convulsions prior to euthanasia.

*Endocrine tissues* — In the adrenal glands of all 6 calves, there was multifocal immunohistochemical staining for BVDV (Fig. 9). This was almost exclusively in the zona reticularis, with only an occasional BVDV-positive, presumably neuroendocrine, cell in the medulla. In one calf (No. 2) there were small foci of necrosis associated with this BVD viral antigen. In the same calf there was focal viral replication in the pituitary gland. There was no evidence of viral replication in the thyroid glands of 5 of 5 calves in which this tissue was evaluated.

*Urinary tract* — There was no evidence of BVDV antigen in the kidneys of these 6 calves. One calf (No. 6395) had a focus of viral antigen in the smooth muscle of the tunica muscularis of the urinary bladder.

*Skin* — Multiple sections of skin consisting of cutaneous and mucocutaneous sites were examined in one calf (No. 6395), and multifocally, there was viral antigen in several layers of squamous epithelium (Fig. 10) and in hair follicles.

*Controls* — There were no remarkable gross or histological lesions in either of the sham-inoculated control calves. Neither was there immunohistochemical staining for BVDV antigen in tissue sections from these calves, nor staining with the irrelevant control monoclonal antibody.

Positive control sections of enteric tissue from a case of mucosal disease had dark granular cytoplasmic staining of cells in the lamina propria and tunica muscularis.

## DISCUSSION

This BVDV type II isolate caused severe and rapidly progressive clinical disease in young seronegative calves. There was variably extensive, multisystemic distribution of BVDV antigen, probably indicative of viral replication in multiple cell types. There were few gross or histological inflammatory lesions. These findings indicate that the virus can induce severe disease, the cause of which may not be apparent on the basis of routine postmortem examination. Hemorrhage, which has come to be associated with acute BVDV type II infections, was not a predominant pathological finding in these calves. Moreover, the common finding of pulmonary consolidation could be interpreted as enzootic pneumonia, in which BVDV would infrequently be considered as an etiologic agent.

Intranasal inoculation of low passage BVDV to colostrum-fed calves, as opposed to intravenous inoculation of infected blood (18) or inoculation following corticosteroid administration (11), was used in this study to approximate more closely the natural transmission of the virus. Moreover, these calves were of the same age as those affected in recent BVDV epidemics in veal calves (3,7). The calves in this study all developed watery diarrhea, high fever and pyrexia, variable anorexia, and severe depression, beginning approximately 7 d after challenge. This is in contrast to another recent study in which 2 noncytopathic BVDV type II isolates caused mild or no disease following inoculation of neonatal specific, pathogen-free calves (10). The differences in clinical presentation likely indicate heterogeneity in virulence of BVDV type II isolates and in the clinical manifestations they produce (10,18,19); however, animal age and management (gnotobiotic versus conventional rearing) could also affect clinical responses and disease expression. These data also indicate that the virulence of isolate No. 24515 is stable through at least 8 passages in cell culture, as is apparently the case with another noncytopathic type II isolate (isolate 890) (20).

The role of acute BVDV infections in respiratory disease in cattle is controversial (3). In part, this is due to the difficulty in differentiating acute versus chronic (persistent) infection in field cases in cattle, including young calves (21,22). In recent experimental infections with 2 BVDV type II isolates, neither pneumonia nor immunohistochemical evidence of pulmonary infection were reported (10). Similarly, in a previous report of experimentally-induced acute infections, BVDV antigen was identified only in peribronchiolar lymphoid tissue and no pulmonary lesions were noted (12). In contrast, as was the case in the herd from which 24515 was isolated (5), calves in the present study consistently had pneumonic lesions at the time of necropsy. These data support the concept that the pneumopathogenicity, and perhaps pulmonary target cell tropism, varies amongst acutely infecting BVDV isolates (23). However, in only one calf in the present study could BVDV infection of bronchiolar epithelium account for the pneumonia. Instead, infection of pulmonary alveolar macrophages was apparent in most of these calves. Our findings are further supportive of the concept that BVDV infection adversely affects the immune surveillance functions of pulmonary alveolar macrophages (PAM) (24–26). It has been demonstrated experimentally that BVDV infection concurrent with *Pasteurella haemolytica* (25) or bovine herpesvirus-1 (24) results in more severe pulmonary disease or more extensive viral replication (BHV-1) than either of the pathogens alone. Currently there is only one report on the effects of postnatal BVDV infections on lung macrophages (26); therefore, specific functional alterations in PAM occurring with BVDV infection warrant further attention.

Viral replication has been reported in various lymphocyte subsets based on immunohistochemical staining of the blood of cattle with mucosal disease (27). Although we and others found that type II BVDV virus can be transiently isolated from buffy coat leukocytes during acute infection (5,10,18,20,28), in the present study there was no convincing immunohistochemical evidence of BVDV type II infection of lymphocytes in lymph nodes, spleen, nor white blood cells in inflamed tissues. This was also apparently the case in gnotobiotic calves infected with BVDV type II isolates of low virulence that did not cause significant clinical disease (10). Extensive and prolonged replication

in differentiated lymphocytes in blood and secondary lymphoid organs may be a feature of persistent infection, or could vary with the age of the animal at the time of infection, stage of infection, or isolate of BVDV.

In contrast to the variable and often minimal evidence of viral antigen in secondary lymphoid organs, there was consistently abundant BVDV antigen in the ileal Peyer's patches and thymus. Ileal Peyer's patches are critical in B-cell development in ruminants (29). Viral antigen was apparent in nonlymphoid "stromal" cells in ileal (and jejunal) Peyer's patches, in which there was depletion of lymphocytes and early involution. Stromal cells comprise subpopulations of fibroblastic reticulum cells, dendritic cells and follicular dendritic cells (30). The phenotype and function of stromal cells is poorly defined at present, and further studies are required to examine the apparent tropism of BVDV for subpopulations of these cells. Although no morphometric analyses were conducted in these calves, thymuses were also considered mildly to moderately depleted of lymphocytes compared to clinically normal calves; however, this depletion was associated with apparent viral replication in stromal cells, not lymphocytes. Mechanisms of lymphoid depletion, as well as potential longterm immunosuppressive implications for calves that survive acute infections, require further investigation.

Changes in the leukograms of the calves in the present study, specifically, neutropenia with a left shift to immature forms and toxic change, is more consistent with a peripheral tissue demand for neutrophils in excess of granulopoiesis, than with myelosuppression alone. However, both mechanisms may have been contributory, as all calves had bone marrow atrophy and, often, necrosis, as well as pulmonary inflammation. Depressed granulopoiesis may have been due to direct effects of BVDV infection in myeloid progenitor cells, or via the indirect effect of virus-induced cytokines (31).

The anemia in the calves in this study was attributed to indirect effects of viral infection, such as hypersecretion of cytokines (31), in as much as BVDV antigen was not detected in erythroid progenitor cells. The mechanism of thrombocytopenia during acute BVDV infections is unresolved. Previously, there has been little direct evidence of infection of megakaryocytes (18,28,32). However, ultrastructural abnormalities of platelets (33) and megakaryocyte degeneration (34) have been reported in swine infected with hog cholera virus. As in another recent study (10) the presence of viral antigen in megakaryocytes in 2 of these calves, including one with hemorrhages at necropsy, suggests that direct infection of precursor cells in the bone marrow may play a role in platelet depletion, and the resultant hemorrhagic syndrome that is a feature of some BVDV type II infections (18,20).

We consistently found viral antigen in the adrenal cortex of these experimentally-infected calves. Histological changes and localization of BVDV in this anatomical site has previously been reported in cattle with mucosal disease (35,36). Moreover, in the present study, BVDV antigen was primarily localized to cells of the zona reticularis. Aside from a high degree of vascularity, it is not apparent why the virus has a tropism for the adrenal gland, or cells of the zona reticularis in particular. There was evidence in only one infected calf of necrosis in foci of viral infection. If infection were associated with hypersecretion of adrenal corticoids, this hyperadrenocorticism could account for some of the hematological findings, such as lymphopenia and marrow necrosis, and may have contributed to clinical disease through electrolyte imbalance.

Smooth muscle in numerous organs, including the tunica media of arterioles, was a common site of viral antigen in these calves, and has previously been reported in cattle with mucosal disease, and in aborted fetuses and neonatal deaths (16,37). In the present study, this immunolocalization occurred without any apparent inflammatory response. To our knowledge, replication of BVDV in smooth muscle has not been previously recognized in animals with acute BVDV infections.

Only one infected calf in this study had any clinical signs referable to the central nervous system. Aside from small foci of viral replication in the pituitary gland, BVDV antigen was not found in the the brain of any of the infected calves. This is in contrast to the often extensive viral replication in nervous and ocular tissues, including ganglia in various organs, in persistently infected cattle that has been causally linked to a wide variety of congenital defects in the nervous system (3,27,35).

Our results are consistent with previous data indicating the tropism of various BVDV isolates for keratinized epithelium (10,17,27,35). There was multifocal viral replication in squamous epithelium of the oral cavity and the forestomachs. Often this occurred without obvious damage to infected cells, and in the absence of an accompanying cellular inflammatory response, as has been reported in clinically "normal," persistently-infected cattle (27). Although BVDV replication is reported in the skin of cattle with mucosal disease (27), viral replication has not been previously reported in this organ in cattle with acute infections.

There was little evidence of viral replication in absorptive mucosal epithelial cells in the small or large intestines that could account for the consistent diarrhea that developed subsequent to BVDV type II infection in these calves. Viral replication has previously been reported in crypt epithelium of persistently-infected, clinically normal cattle with mucosal disease, and, less extensively in cattle with acute infections (10,17,27,38). Extensive infection of enteric mucosal epithelium with noncytopathic BVDV may principally occur in persistently-infected cattle, in which there is often extensive extracryptal replication of cytopathic biotypes in the intestinal mucosae (38), as well.

Aside from mild to moderate pneumonic lesions in all calves, there were few gross or histological lesions to account for the severity of clinical disease in these calves; therefore, the mechanisms of the clinical signs observed, including, diarrhea, pyrexia, and depression are unresolved. As in previous studies of cattle with mucosal disease, we found consistent systemic replication of BVDV in cells of monocyte/macrophage lineage, including dendritic cells in lymphoid organs. Although there are data documenting the effect of cytokine

secretion on BVDV replication in fibroblasts in vitro (39), there is virtually nothing known regarding the effects of BVDV on the cytokine secretory functions of infected macrophages, in vitro or in vivo. Virus stimulated hypersecretion of cytokines (or corticosteroids), such as interferon α, TNF-α, and IL-1, could have adverse pleiotropic physiologic (40) effects resulting in disease similar to that observed in these calves. This possibility warrants further study, since it would appear that the host response to viral infection, rather than direct effects of the virus per se, may be responsible for clinical disease.

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